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Analysis of the Role of Flagella in the Heat-Labile Lior Serotyping Scheme of Thermophilic Campylobacters by Mutant Allele Exchange

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Flagellin mutations originally constructed in *Campylobacter coli* VC167 (serotype LIO8) by a gene replacement mutagenesis technique (P. Guerry, S. M. Logan, S. Thornton, and T. J. Trust, J. Bacteriol. 172:1853–1860, 1990) were moved from the original host into *Campylobacter* strains of a number of other Lior serogroups by a natural transformation procedure. This is the first report of the use of this transformation method to transfer a mutated locus among *Campylobacter* strains. Flagellin mutants were constructed in a number of heat-labile LIO serotypes and were serotyped and analyzed by immunoelectron microscopy with LIO typing antisera. In six cases, isogenic nonflagellated mutants were able to be serotyped in the same serogroup as their parent, and immunogold electron microscopy confirmed that antibodies in the typing antisera bound to components on the surface of both parent and mutant cells. However, in only one case, a strain belonging to serogroup LIO4, was a nonflagellated mutant untypeable, and immunogold electron microscopy showed that antibodies bound to the flagella filament of the parent but not to the cell surface. Furthermore, after introduction and expression as a flagellar filament of a LIO8 flagellin gene in this mutant, the strain could not be serotyped. These results indicate that a nonflagellar antigen is often the serodeterminant in the heat-labile Lior serotyping scheme.

The recognition of *Campylobacter jejuni* and, to a lesser extent, *Campylobacter coli* as major causes of bacterial gastroenteritis worldwide has resulted in considerable interest regarding the epidemiology of the disease (3, 4, 31). In order to fully understand the epidemiological significance of *Campylobacter* infections, a number of different serological testing schemes have been independently developed. The two most commonly used schemes are based on heat-stable and heat-labile antigens; however, there are others that use direct immunofluorescence (10–12) or coagglutination (5, 14, 37).

Initially, Berg et al. (2) presented evidence that *Campylobacter* strains carry both thermostable and thermolabile antigens. Two independent groups, Penner and Hennessy (27) and Lauwers et al. (16), developed serotyping schemes on the basis of soluble, heat-stable antigenic factors. Both schemes used the passive hemagglutination technique with unabsorbed antisera (28). It has been shown that the serospecific determinant of this widely used thermostable typing scheme is the lipopolysaccharide (23, 29).

In the case of the heat-labile antigen, Lior et al. (18) developed a slide agglutination serotyping scheme to differentiate thermophilic campylobacters on the basis of heat-labile antigenic factors. This widely used scheme involved the use of live whole cells as the antigenic material and typing antisera that had been absorbed with heat-stable preparations of the homologous serostrain. Further absorptions were performed, if necessary, with heat-labile antigens of cross-reacting heterologous strains. This yielded monospecific antisera to the heat-labile antigens of the homologous serostrain. Similar typing schemes have been developed by Rogol et al. (30) and Itoh et al. (15). The precise molecular nature of the serodeterminants in the heat-labile typing schemes have not, as yet, been fully elucidated.

The polar flagellum possessed by the *Campylobacter* cell not only imparts motility, allowing effective colonization of the intestinal tract (17), but also appears to be a major protein antigen on the cell surface (20, 21, 24). Furthermore, flagella have been suggested to be the serodeterminant in four of the Lior serotypes, LIO5, LIO6, LIO7, and LIO17 (35, 36). The Lior heat-labile serotyping scheme recognizes more than 100 serogroups (19), which suggests a high degree of antigenic diversity among the serodeterminants. However, *Campylobacter* flagellins have been shown by Western blot (immunoblot) analysis to possess antigenic cross-reactivity (21), although specific surface-exposed epitopes have also been reported (6, 9). Furthermore, among 20 serotypes that were examined (32), the two tandemly orientated flagellin genes present in the *C. coli* VC167 chromosome, *flaA* and *flaB*, possess significant overall DNA homology, although this homology resides in the 5′ and 3′ regions of the gene. A probe derived from the central region of the VC167 flagellin gene was specific for organisms belonging to the LIO8 serogroup (32). It has been shown that both FlaA and FlaB flagellins are present in the flagellar filament of VC167, although FlaB is present in significantly smaller quantities than FlaA (6). In the absence of the *flaA* gene product, the *flaB* gene product has been shown to produce a truncated filament on the surface of the cell that is functional, yet that results in greatly reduced motility (6). Nucleotide sequence analysis of the flagellin genes from *C. jejuni* 81116 also revealed the presence of the *flaA* and *flaB* genes (25). However, the role of two flagellin genes in *C. jejuni* has been

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TABLE 1. Strains used and mutants constructed in this study

Strain	Donor DNA[a]	Flagella[b]	Motility	Serotype
C. coli VC167	NA[c]	Full length	+++	8
C. coli VC167B2	NA	Absent	−	8
C. coli VC167B3	NA	Absent	−	8
C. coli VC20	NA	Full length	+++	8
C. coli VC20K	VC167B2	Truncated	+	8
C. jejuni VC152	NA	Full length	+++	8
C. jejuni VC152K	VC167B2	Truncated	+	8
C. coli VC97	NA	Full length	+++	20
C. coli VC97K	VC167B2	Truncated	+	20
C. coli VC97-3	VC167B3	Absent	−	20
C. jejuni VC103	NA	Full length	+++	17
C. jejuni VC103K	VC167B2	Absent	−	17
C. jejuni VC104	NA	Full length	+++	19
C. jejuni VC104K	VC167B2	Absent	−	19
C. jejuni VC87	NA	Full length	+++	1
C. jejuni VC87K	VC167B2	Absent	−	1
C. jejuni VC91	NA	Full length	+++	11
C. jejuni VC91K	VC167B2	Truncated	+	11
C. jejuni VC83	NA	Full length	+++	4
C. jejuni VC83K	VC167B2	Absent	−	UT[d]
C. jejuni VC83/KX5	VC167/KX5	Full length	+++	UT
C. jejuni VC84	NA	Full length	+++	6
C. jejuni VC84K	VC167B2	Truncated	+	6
C. jejuni VC84/KX5	VC167/KX5	More than full length	+++	6
C. jejuni VC84-1	VC167B3	Absent	−	6

[a] Genomic DNA used in transformation procedure.
[b] As determined by electron microscopy.
[c] NA, not applicable.
[d] UT, untypeable.

obscure, because Nuijten and coworkers (26) failed to detect expression of the *flaB* gene.

In this study, we attempted to determine the role of the flagella antigen in representative strains of the Lior typing scheme. By using the substantial DNA homology among *Campylobacter* flagellin genes (32), mutants were constructed in a number of Lior serotypes by a natural transformation method (34), representing the first instance of mutant allele exchange among *Campylobacter* strains, and we report our findings here.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacteria used in this study were *C. coli* VC167, VC97, and VC20 and *C. jejuni* VC84, VC91, VC83, VC87, VC152, VC103, and VC104 from the University of Victoria collection and are described in Table 1 (H. Lior, National Enteric Reference Centre, Ottawa, Ontario, Canada). The flagellin mutants *C. coli* VC167B2, VC167B3 (7), and KX5 (6) were from this laboratory. Stock cultures were maintained at −70°C in 32% (vol/vol) glycerol and 0.6% (wt/vol) Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.). Cultures were grown on Mueller-Hinton agar (Oxoid Ltd. Basingstoke, United Kingdom) at 37°C in an atmosphere containing 5% CO_2. Agar was supplemented with 100 μg of kanamycin (Sigma) per ml, when required, including during serotype analysis. Motility was tested on agar plates made with Mueller-Hinton broth supplemented with 0.4% Bacto Agar (Gibco) and was assessed quantitatively by measuring the radius of bacterial growth.

Construction of flagellin mutants. Flagellin mutants were constructed by following the biphasic transformation method described by Wang and Taylor (34) by using 4 μg genomic DNAs of the *C. coli* VC167B2, VC167B3, or KX5 mutants described previously by Guerry et al. (6, 7). Selection of transformants was on Mueller-Hinton agar supplemented with 100 μg of kanamycin per ml.

DNA extraction and hybridization. Total DNA extractions from *Campylobacter* cells were achieved by the method of Hull et al. (13). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) or Pharmacia (Uppsala, Sweden) and were used under the conditions recommended by the suppliers. DNA was nick translated with [α-^{32}P]dCTP by using a commercial kit (Dupont, NEN Research Products, Boston, Mass.). Conditions for hybridization were as described previously (8).

Electron microscopy. Samples were negatively stained with a solution of 1% ammonium molybdate and 0.1% glycerol (pH 7.0) on Formvar carbon-coated grids and were examined on either a Phillips EM300 or a JEOL electron microscope. For immunoelectron microscopy, bacterial cells on Formvar carbon-coated grids were incubated with 0.2% bovine serum albumin in TBS (10 mM Tris-HCl, 0.9% NaCl [pH 8.0]) for 30 min; this was followed by an incubation with a 1:20 dilution of the LIO typing antiserum for 60 min at room temperature. After three washes with TBS, the grids were incubated for a further 60 min with 1:50 dilution in TBS of protein A-colloidal gold (diameter, 15 nm; Jannsen Pharmaceutica, Olen, Belgium). After an additional five washes in TBS, the cells were negatively stained and examined as described above.

Serotyping. The cultures were serotyped by slide agglutination as described previously (18). Mutant strains were grown on Mueller-Hinton agar supplemented with 100 μg of kanamycin per ml. The strains were examined by electron microscopy before and after serotype analysis.

RESULTS

Construction and characterization of the flagella mutants. The original flagellin mutations in strain VC167 were generated by insertion of a kanamycin resistance cassette into a cloned flagellin gene on a suicide vector, followed by conjugal transfer from *Escherichia coli* into VC167. The mutated flagellin gene was rescued by homologous recombination into the chromosome, resulting in defined mutations in either the *flaA* gene (VC167B2) (Fig. 1A) (7) or the *flaB* gene (KX5) (Fig. 1A) (6) or in a deletion involving part of the *flaA* and the *flaB* genes (VC167B3) (Fig. 1A) (7). Since there is a high degree of overall homology among flagellin genes of *C. coli* and *C. jejuni* (32), we attempted to move these defined flagellin mutations into other strains of both *C. coli* and *C. jejuni* by means of a natural transformation procedure (34). Genomic DNAs from the three flagellin mutants of VC167 were used to transform *Campylobacter* strains of various LIO serotypes to kanamycin resistance. The strains used and the mutants generated are given in Table 1.

The frequency of kanamycin-resistant transformants varied among the strains tested but ranged from 2.5×10^{-6} to 4

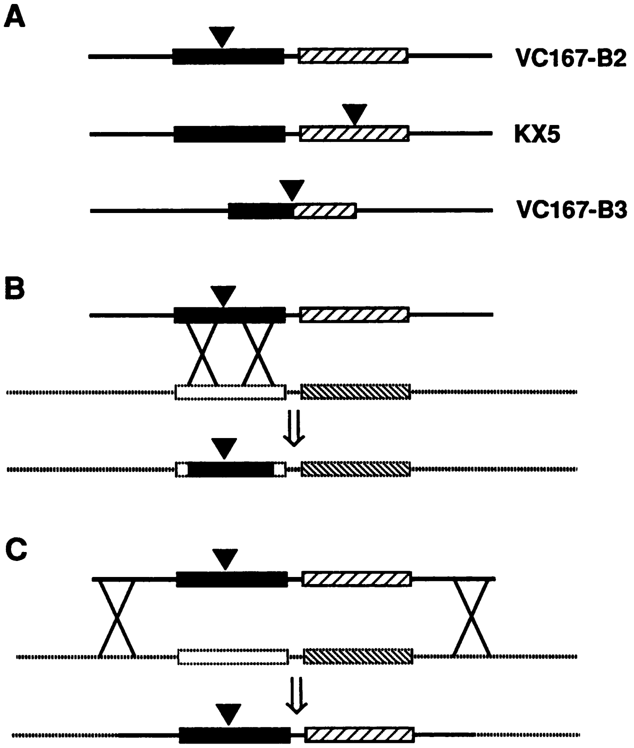


FIG. 1. (A) Schematic representation of the flagellin genes of the three VC167 mutants whose DNAs were used to transform other *Campylobacter* strains. The solid black shading represents the *flaA* gene, and the hatched shading represents the *flaB* gene. The black triangle represents the kanamycin resistance cassette which was inserted in vitro into the VC167 flagellin genes (7). (B) and (C) Schematic representations of two classes of crossover events between a donor DNA (in this case, VC167B2) on the top line and the resident flagellin genes on the chromosome of the recipient strain. In panel B, the crossover occurs within one flagellin gene, resulting in one mutated flagellin gene and one intact resident flagellin gene. In panel C, the crossover occurs at homologous sequences outside of the flagellin gene cluster, resulting in a total replacement of the resident flagellin genes with those of the donor.

$\times 10^{-5}$. Transformants were tested for motility on semisolid agar, and selected transformants were examined for their flagellar structures by electron microscopy, and their flagellin genotypes were determined by Southern blot analysis (6). Both VC167B2 (*flaA flaB*$^+$) and VC167B3 (*flaA flaB*) mutants produced no flagellar filament, although VC167B2 had detectable levels of flagellin product intracellularly (7). However, it has recently been shown that other *flaA flaB*$^+$ mutants of VC167 can produce truncated flagellar filaments on the cell and are motile, although much less so than wild-type cells (1, 6). Mutants constructed by using VC167B2 DNA displayed two phenotypes. Some (VC83K, VC87K, VC103K, and VC104K) were bald, like VC167B2, and others (VC84K, VC97K, VC91K, VC20K, and VC152K) possessed the truncated flagellar filament and slight motility characteristic of other *flaA flaB*$^+$ mutants (1, 6). The truncated filament of mutant VC84 can be seen compared with the full-length wild-type filament of VC84 (Fig. 2). Genomic DNAs were digested with the restriction enzyme *Ssp*I, electrophoresed on 0.7% agarose gels, and transferred to nitrocellulose membranes. The membranes were probed with pGK213 (6), which is full-length flagellin probe which hybridizes to all *C. jejuni* and *C. coli* strains tested (32), and/or pGK209, which is an internal flagellin probe shown to be specific for LIO8 strains (32). Figure 3A shows the pattern of hybridization of pGK213 to wild-type VC167 (lane 1). The top band in lane 1, which is 2.3 kb, includes all of the *flaA* gene, and the bottom 1.6-kb band corresponds to most of the *flaB* gene (6). Lane 2 shows the pattern of hybridization of VC167B2, in which the *flaA* sequence is disrupted by insertion of the kanamycin resistance gene, which contains an internal *Ssp*I site (6, 33). The pattern of strain VC84K, a mutant of strain VC84 obtained by using VC167B2 DNA, is shown in lane 5 and can be compared with the wild-type VC84 pattern in lane 4. A pattern similar to that of strain VC167B2 is seen, although some VC84 flagellin sequences seem to have been conserved. This DNA was further analyzed by hybridization with pGK209, and these results are given in Fig. 3B. This probe hybridized strongly to VC167 (lane 1), VC167B2 (lane 2), and VC167B3 (lane 3), but not to VC84 (lane 4) or to the VC84K mutant (lane 5), suggesting that little VC167 flagellin information remained in this mutant. A similar result is seen in Fig. 3A for VC91K (lane 8), a mutant of VC91 (lane 7) obtained with VC167B2 DNA, which also produces a truncated filament. Hybridization of this DNA with pGK209 also confirms that little, if any, VC167 flagellin information is present in VC91K (data not shown). Similar results were obtained with VC97K, suggesting that the truncated filament produced in these mutants is probably encoded by a resident *flaB* gene and not that of VC167. The presence of VC167B2 flagellin information in the VC20K and VC152K mutants could not be determined since these two strains are LIO8, and their flagellin genes hybridize strongly with VC167 (32). In the case of the four bald mutants generated with VC167B2, VC167 flagellin information could be detected in only one (VC83K).

Both the mutants constructed by using VC167B3 DNA (VC97-3 and VC84-1) possessed, as expected, a bald phenotype (Fig. 2c and Table 1) and were nonmotile. The hybridization pattern of the deletion mutant VC167B3 is shown in Fig. 3A, lane 3. The hybridization pattern of VC84 wild-type DNA is shown in lane 4. VC84-1, a transformation mutant obtained by using DNA from VC167B3, showed a hybridization pattern identical to that of VC167B3 (lane 6) rather than to that of the VC84 parent. A similar hybridization pattern was seen with VC97-3 (data not shown).

Mutant KX5 (*flaA*$^+$ *flaB*) is fully motile and produces a flagellar filament indistinguishable in length from that of wild-type VC167 (6). Figure 3C shows hybridization of pGK209 to VC167 (lane 1), KX5 (lane 2), VC84 (lane 3), and mutant VC84/KX5 constructed with KX5 DNA (lane 4). In this case, and that of VC83/KX5 (data not shown), KX5 genetic information was clearly added to the resident chromosomes. Both VC83/KX5 and VC84/KX5 mutants were motile. VC83/KX5 produced a flagellar filament indistinguishable in length from that of a wild-type filament, but VC84/KX5 produced a filament longer than the wild-type cell (Fig. 2d). Furthermore, extra-long flagellar filaments produced by VC84/KX5 (Fig. 2e) could often be detected. These long filaments would be highly susceptible to breakage, and may have accounted for the higher amount of broken flagellar filaments seen per field of vision for strain VC84/KX5 when compared with the amount seen for strain VC84 (data not shown). However, motility testing revealed that there was no difference in the degree of motility between the VC84 wild type and VC84/KX5, despite this difference in filament length.

Southern blot analysis indicated that two major classes of

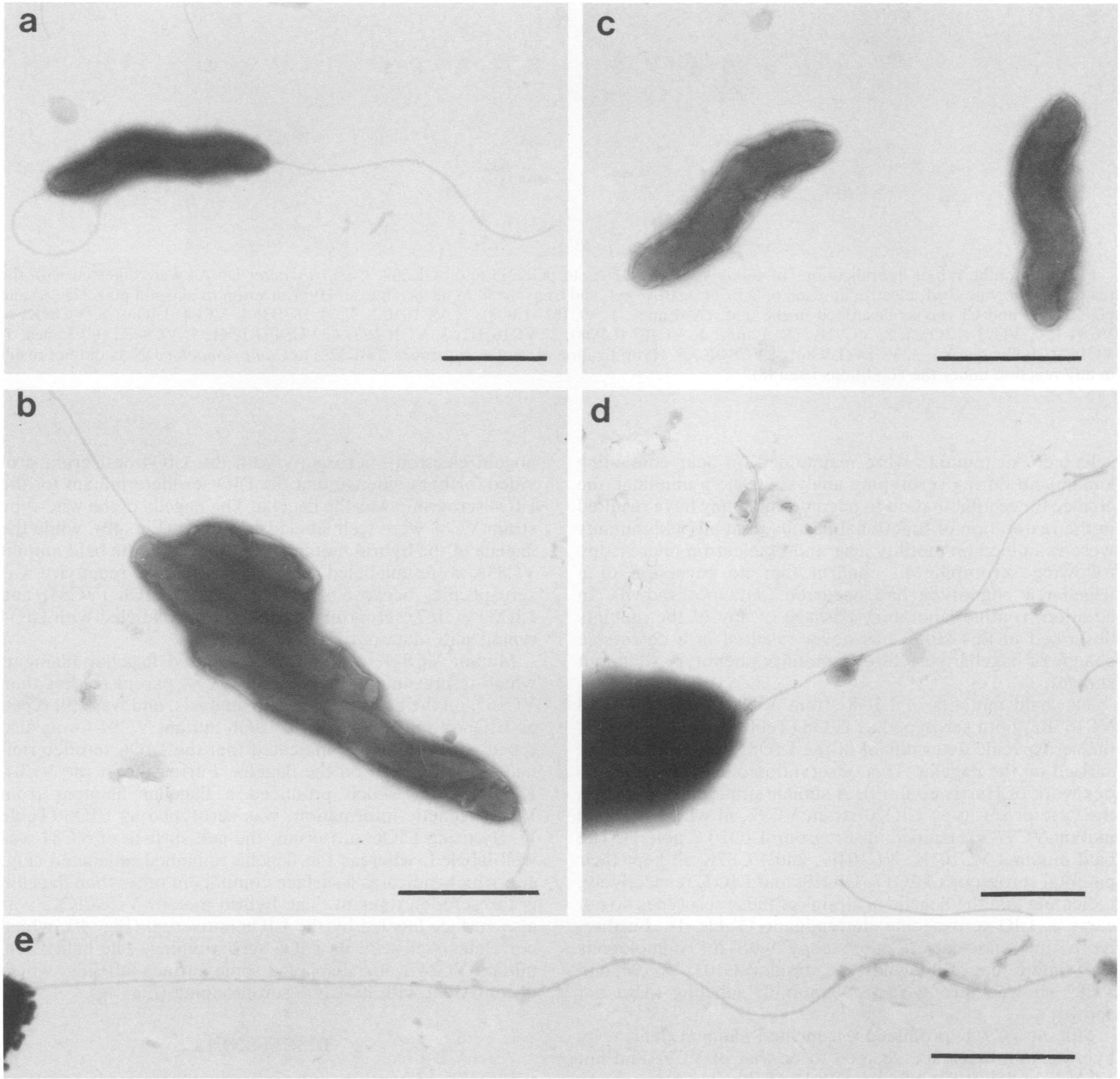


FIG. 2. Electron micrographs of negatively stained VC84 (a), the *flaA flaB*$^+$ mutant VC84K producing a truncated flagellar filament (b), the bald *flaA flaB* mutant VC84-1 (c), VC84/KX5 (d), and VC84/KX5 producing an extra-long flagellar filament (e). Bars, 1 μm.

recombinational events occurred, as depicted in Fig. 1B and C. In the first class (Fig. 1B), the crossover event occurred at some point within a flagellin gene, such that the kanamycin resistance cassette and an undetermined amount of VC167 flagellin sequence crossed over into a resident flagellin gene. In the example shown in Fig. 1B, the donor DNA was VC167B2, in which the kanamycin cassette and adjacent sequences within the *flaA* gene crossed over into the resident *flaA* gene and the resident *flaB* gene remained intact. This class of recombinant was exemplified by mutant VC84K, which produced a truncated flagellar filament presumably encoded by its own *flaB* gene. Figure 1C shows a recombinational event in which a crossover event between donor DNAs occurred at sites outside of the flagellin gene cluster, resulting in a total replacement of the resident flagellin information for that of donor DNA. This class of recombinant is exemplified by mutant VC84-1 (in which the donor DNA was VC167B3) and VC84/KX5 (in which the donor DNA was KX5).

Effect of flagella mutation in serotyping. The mutants constructed in this study were serotyped by the heat-labile Lior serotyping scheme and were examined by immunoelectron microscopy to analyze the role of flagella in the serospecificity of the representative LIO serogroups that were

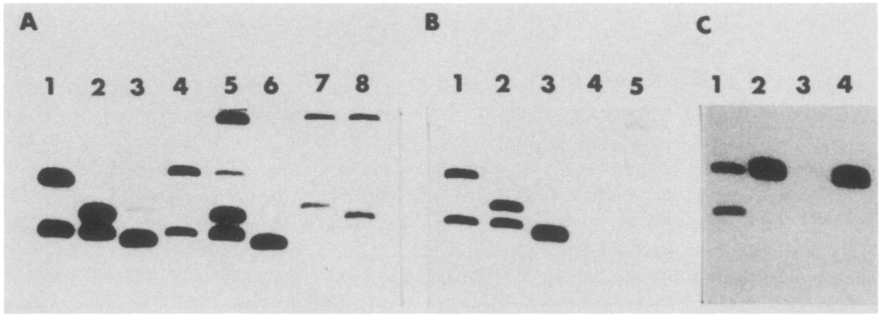


FIG. 3. Southern blot hybridizations of *Campylobacter* DNAs to pGK213 and pGK209. *Campylobacter* DNAs were digested with the restriction enzyme *Ssp*I, electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose. Hybridization to plasmid pGK213 (A) and pGK209 (B and C) was as described in the text. (A) Lanes: 1, VC167 (LIO8); 2, VC167B2; 3, VC167B3; 4, VC84 (LIO6); 5, VC84K; 6, VC84-1; 7, VC91 (LIO11); 8, VC91K. (B) Lanes: 1, VC167 (LIO8); 2, VC167B2; 3, VC167B3; 4, VC84 (LIO6); 5, VC84K. (C) Lanes: 1, VC167 (LIO8); 2, KX5; 3, VC84 (LIO6); 4, VC84/KX5. Hybridization of vector sequences (pBR322) to *Campylobacter* DNAs did not result in any reaction under the conditions used (8).

selected. All mutants were maintained on agar containing kanamycin during serotyping analysis to help minimize the chance for recombination to occur, which may have resulted in the restoration of functional flagella, and all bald mutants were examined on motility agar and by electron microscopy following serotyping to confirm that no reversion to a flagellated phenotype had occurred (data not shown). In addition, routine laboratory passage of any of the mutants described in this study has never resulted in a detectable change of flagellar structure or motility phenotype (data not shown).

The bald mutants of LIO8 strain VC167, VC167B2 and VC167B3, both serotyped as LIO8 (Table 1), indicating that the serospecific determinant of the LIO8 serogroup was not carried on the flagella. This observation confirms the previous work of Harris et al. (9). A similar situation was seen in the case of serotype LIO20 strain VC97, in which the bald mutant VC97-3 remained in serogroup LIO20 (Table 1). The bald mutants VC103K, VC104K, and VC87K all kept their parental serogroups LIO17, LIO19, and LIO1, respectively, indicating that the flagella of strains of these serotypes do not carry a LIO-serospecific determinant (Table 1). Furthermore, immunoelectron microscopy with the homologous LIO typing sera of the wild-type strains VC103, VC104, and VC87 showed strong surface antibody labeling (data not shown).

Mutant VC91K produced a truncated filament, which, by hybridization analysis, seemed to be that of VC91 and not VC167, but it still typed as LIO11 (Table 1). Attempts to produce a bald mutant of VC91 by transformation with VC167B3 DNA was repeatedly unsuccessful, because a "stubby" flagellum phenotype was always obtained. However, by immunogold electron microscopy VC91 and VC91K showed heavy surface antibody labeling with the LIO11 antiserum and no flagellar filament labeling (Fig. 4a, data not shown), implicating a surface component other than flagellin as the LIO11 serospecific antigen.

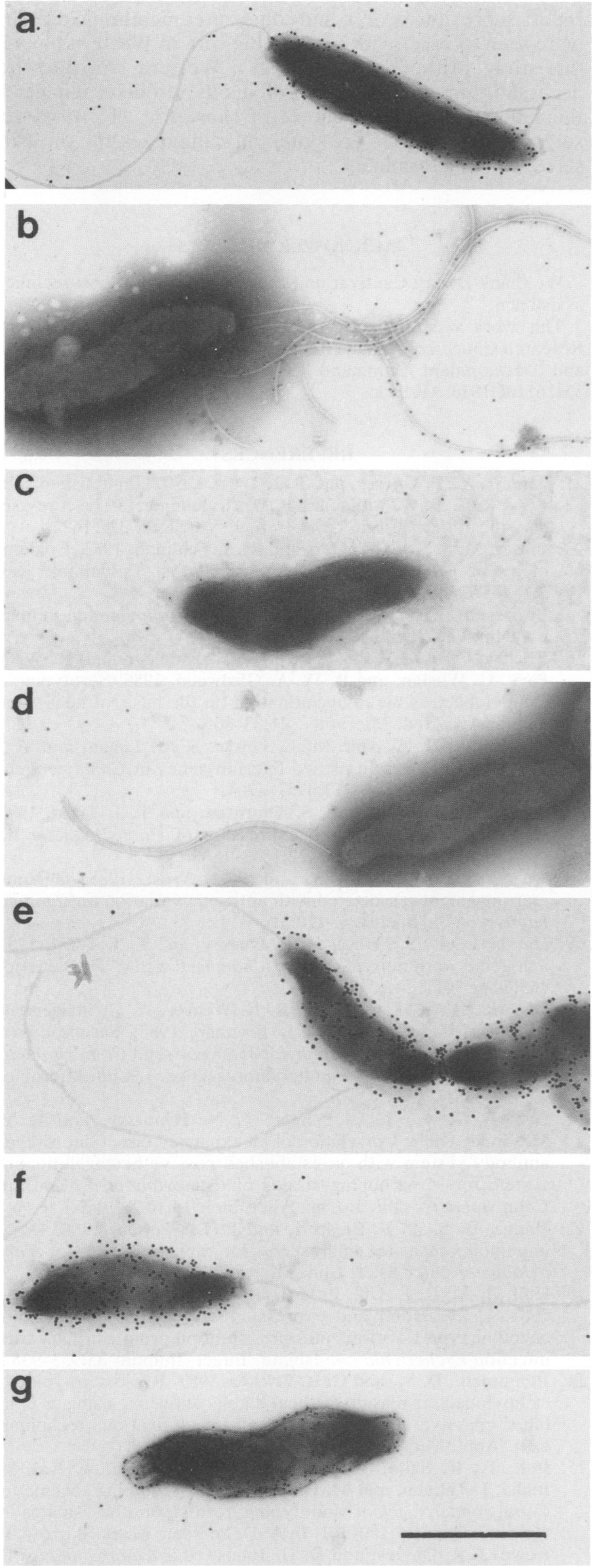
In contrast to these findings, the bald mutant of VC83, VC83K, was not typeable (Table 1). This indicated that flagellin is the serodeterminant of the LIO4 serogroup. Hybrid strains of VC83 were also constructed by using DNA from mutant KX5 (6) in order to express a flagellum belonging to another serotype. This hybrid VC83/KX5, although producing a flagellar filament composed of the *flaA* gene product of VC167, was also untypeable (Table 1). Immunogold electron microscopy with the LIO4 antiserum provided further evidence that the LIO serodeterminant for the LIO4 serogroup was the flagella. The flagella of the wild-type strain VC83 were well labeled with gold (Fig. 4b), while the flagella of the hybrid mutant VC83/KX5 and the bald mutant VC83K were unlabeled (Fig. 4c and d). This reactivity was serospecific, because strains from the LIO6 (VC84) and LIO8 (VC167) serogroups were totally unlabeled with LIO4 typing sera (data not shown).

Mutant VC84K produced a truncated flagellar filament, which is presumably that of the VC84 parent, rather than VC167, on the basis of Southern analysis, and was still typed as LIO6 (Table 1). The bald LIO6 mutant VC84-1 was also typed as LIO6, which indicated that the LIO6 serodeterminant is not carried on the flagella. Furthermore, the VC84/KX5 mutant, which produced a flagellar filament from VC167 genetic information, was serotyped as LIO6 (Table 1). By using LIO6 antiserum, the cell surface of VC84 was well labeled, whereas the flagella remained unlabeled (Fig. 4e), which indicates a surface component other than flagellin is the serodeterminant. The hybrid mutant VC84/KX5 was also surface labeled by the LIO6 antiserum (Fig. 4f), which correlates well with its LIO6 serogrouping. The bald LIO6 mutant VC84-1 also displayed some surface labeling, which is consistent with its LIO6 serogrouping (Fig. 4g).

DISCUSSION

Flagellin mutants were genetically constructed in isolates from a variety of different serotypes by means of mutant allele exchange via homologous recombination. These experiments with mutated flagellin genes from *C. coli* VC167B2 (*flaA flaB*$^+$), VC167B3 (*flaA flaB*), and KX5 (*flaA*$^+$ *flaB*) mark the first exploitation of the previously observed natural transformability of campylobacters for genetic analysis. From this perspective, the transfer of the mutated VC167 flagellin genes into alternate hosts has generated interesting and important information on the regulation of flagella and the role that this antigen plays in the heat-labile Lior serotyping scheme.

With two exceptions, the flagellin mutants constructed in this study were classified by electron microscopic examination either as producing short truncated stubby flagella or as bald, that is, not producing any detectable flagellar filament on the cell surface. The two exceptions were the two



mutants constructed from *flaA*$^+$ *flaB* KX5 DNA. One mutant (VC83/KX5) displayed a filament of normal length, while the other (VC84/KX5) produced an unusually long filament, suggesting the possibility that the crossover event in this mutant may have disrupted an adjacent regulatory locus. The aberrant length of VC84/KX5 is particularly intriguing, since no locus affecting flagella length has been identified in members of the family *Enterobacteriaceae* (22).

In the case of *flaA flaB*$^+$ VC167B2, the original VC167B2 mutation resulted in the *flaB* product remaining intracellular. This appears to be a unique mutation, because all subsequent *flaA* mutations generated in VC167 produced a truncated flagellar filament that was composed exclusively of the *flaB* gene product and that retained some degree of motility (1, 6). Presumably, the inability of the original VC167B2 mutant to produce any truncated filament reflects some secondary defect in flagellin export in this mutant (7). Indeed, the VC83K mutant generated from VC167B2 DNA, in which the VC167B2 *flaB* gene replaced the recipient *flaB* gene, displayed a nonflagellated phenotype, suggesting that the defect in VC167B2 responsible for the intracellular accumulation of *flaB* gene product maps either within the *flaB* structural gene or at another locus very close to the structural gene. In the case of the two mutants which were obtained by transformation with VC167B2 DNA and which were capable of producing a truncated filament characteristic of a *flaB* filament (*C. coli* VC97K and *C. jejuni* VC84K) (6), genetic analyses indicated that in both cases the truncated filament was encoded by the *flaB* gene of the parent strain rather than the *flaB* gene of VC167B2. Importantly, mutant VC84K represents the first indication that *C. jejuni* strains, and not just *C. coli* strains, can express a *flaB* flagellin. Other workers have reported that in another *C. jejuni* LIO6 strain, 81116, the *flaB* gene is present but is not expressed (26).

The bald mutant VC83K of the serogroup LIO4 strain examined in this study could not be typed, implicating flagellar epitopes in LIO4 serotype specificity. This notion was further substantiated by immunogold studies in which the VC83 flagella were well decorated with gold particles, yet the surface remained relatively unlabeled. Upon introduction of a flagellin gene from a LIO8 organism (KX5), a filament was produced that was not labeled by LIO4 antiserum, and the cells could not be typed in the Lior scheme. These data provide solid evidence that in the case of serogroup LIO4, serospecific antigenic epitopes are exposed on the native flagella of the strains.

However, in the majority of Lior serogroups tested (LIO1, -6, -8, -17, -19, and -20), it was demonstrated that the flagella did not carry the LIO serotype determinant. The LIO11 mutant which produced a truncated filament, VC91K, also remained in serotype 11, and Southern blot analysis indicated that the flagellin information contained in this strain was not derived from the donor LIO8 organism. Repeated transformations with VC167B3 failed to give the expected bald phenotype, as the transformants always produced a truncated flagellar filament. However, immunogold analysis of VC91 and VC91K with LIO11 typing sera showed strong

FIG. 4. Immunoelectron microscopy of LIO strains and flagella mutants with serotyping antisera. Strain VC91 reacted with LIO11 antiserum (a); VC83 (b), VC83K (c), and VC83/KX5 (d) reacted with LIO4 typing sera; and VC84 (e), VC84/KX5 (f), and VC84-1 (g) were incubated with LIO6 antiserum Bar, 1 μm.

surface labeling, although the flagella remained undecorated by antibody-protein A gold, again suggesting that a surface component other than the flagella carries the LIO11 serodeterminant.

In the case of serogroup LIO17, the bald mutant VC103K also serotyped in its parental serogroup. This finding is in conflict with a previous report that the flagellar protein is an essential determinant in this serogroup (36). However, those workers used UV irradiation as a means of generating nonflagellated mutants, and it should be recognized that this method of obtaining mutants could create difficulties, because secondary mutations may have occurred. Previous studies also demonstrated that a nonflagellated phase variant of a LIO6 organism, *C. jejuni* 81116, could not be typed in the heat-labile Lior typing scheme (35, 36). In our hands, *C. jejuni* 81116 displaying both a flagellated and nonflagellated phenotype and 81116/KX5 carrying flagellin information from KX5 (LIO8) each bound LIO6 typing sera on the cell surface (data not shown). In both the flagellated strains 81116 and 81116/KX5, the flagellar filaments were unlabeled by LIO6 sera (data not shown). These data confirm the results we obtained with another LIO6 strain, *C. jejuni* VC84. Mutant VC84/KX5 and the bald mutant VC84-1 both typed as LIO6, and immunoelectron microscopy showed that antibodies in the LIO6 antiserum bound to the cell surface of the parent and mutants, indicating that a surface component other than flagella is the serodeterminant of the LIO6 serogroup. In this regard, it is interesting that the binding of antibody-gold complexes to the surface of VC84 (Fig. 4e) and VC84/KX5 (Fig. 4f) appears to be peripheral to the cell envelope, perhaps to a capsule-like antigen. In contrast, the labeling of the bald mutant VC84-1 (Fig. 4g) appears to directly involve the cell envelope. Phase variation of *Campylobacter* flagella has been shown to involve transcriptional regulation of the σ^{28} promoter controlling the *flaA* gene (25). Since nonflagellated phase variants are untypeable (36) but isogenic flagellin mutants are typeable, phase variation may involve coordinate regulation of flagellin and the Lior serodeterminant. This possibility, together with the nature of the LIO6 serodeterminant, is being investigated.

In contrast to the widely held belief that flagella are the determinants of Lior serospecificity, the results presented in this study underscore the complexity of the LIO serotyping scheme. In only one of eight serogroups examined were flagella found to be the Lior serodeterminant (LIO4), and in most cases, flagella have clearly been shown to not be involved in this serotyping scheme (LIO1, -6, -8, -17, -19, and -20). Even though the flagella do not seem to be involved in Lior specificity in most cases, it should be noted that flagella are highly immunogenic (20), and serospecific, surface-exposed determinants on flagella have been demonstrated (6, 9). Indeed, the overall structure of *Campylobacter* flagellin genes resembles that of flagellin genes of members of the family *Enterobacteriaceae* in that the 5′ and 3′ regions of the gene are highly conserved, and the central region, which, in the *Enterobacteriaceae*, encodes H-antigen specificity, is highly variable (32). It is interesting that, at least in the case of LIO8 strains, the central region of this flagellin gene hybridizes specifically to LIO8 strains and not to strains of 20 other Lior serogroups (32), even though flagella are not the LIO8 serodeterminant. This suggests that within a given LIO serogroup flagellin genes are highly conserved, and it would also explain that fact that monoclonal antibodies against *Campylobacter* flagellin can appear to show Lior serospecificity (24). The identities of the serodeterminants in most Lior serogroups remain to be determined. Serospecificity in some outer membrane proteins has been reported previously (20), and some outer membrane proteins were seen to react with Lior typing sera in Western blots in this study. Although reaction in a Western blot does not necessarily implicate these proteins as serodeterminants in the slide agglutination scheme of Lior et al. (18), they are serospecific and thus are potential candidates for the Lior serospecific determinant.

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